Enhanced transduction of fibroblasts in transplanted kidney with an adenovirus having an RGD motif in the HI loop

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Application of gene therapy to the renal graft has a powerful potential to improve the outcome of kidney transplantation and eliminate detrimental side effects associated with systemic therapy, through local expression of immunoregulatory or other protective molecules. However, the search for the optimal vector is still ongoing. In this study, we used a modified adenovirus that has an Arg-Gly-Asp (RGD) motif inserted in the HI loop of the fiber knob, as a successful strategy to transduce the renal graft. Donor Lewis rat kidneys were infused via the renal artery with a solution containing either a fiber-modified adenovirus (AdTL-RGD) or an unmodified adenovirus (AdTL), or with saline. Syngeneic recipients were killed after 3, 7 or 14 days. Efficiency, selectivity, localization, time course of gene expression and side effects were studied using biochemical and immunohistological techniques. Enhanced gene expression was achieved selectively in the transplanted kidney by AdTL-RGD, when compared to AdTL. Transgene expression lasted for at least 2 weeks. With the AdTL-RGD vector, the transgene was abundantly expressed in the renal interstitial fibroblasts. An increase in the number of cytotoxic T lymphocytes accompanied the use of either vector, when compared to saline. These data convincingly show enhanced and selective gene transfer to the fibroblasts of transplanted kidneys using an RGD-modified adenovirus, providing a highly efficient vector system for future therapeutic interventions.

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In humans, the ultimate survival of transplanted kidneys depends largely on extensive immunosuppression. Despite the development of various novel immunosuppressive drugs,[1] side effects associated with systemic immunosuppression, such as opportunistic infections and malignancies, are still major drawbacks in renal transplantation. As a consequence, these life-threatening side effects have to be balanced with effectiveness of the immunosuppression regimens. A promising new strategy to overcome these problems is induction of local immunosuppression in the donor organ by means of gene therapy. In addition, gene therapy approaches could be used to suppress other serious transplantation-related pathological processes, such as ischemia–reperfusion injury and fibrosis.

The transplanted kidney is a particularly appropriate candidate for gene therapy because of its accessibility. However, the lack of a highly efficient, selective and nontoxic vector system has hampered the development of a clinically applicable therapeutic strategy preventing renal transplant failure. Among the nonviral and viral vector systems currently available (reviewed in Van der Wouden *et al.*[2]), the adenovirus has a privileged place, mostly because of its potential to transduce a broad panel of cells. Moreover, when used in the transplantation setting, the adenovirus has the additional advantage of being able to infect several cell types at low temperature, allowing pretransplantation gene transfer to be carried out during the period of cold preservation.[3] Adenovirus targets cells via binding of the fiber knob to the Coxsackie adenovirus receptor (CAR). After binding, internalization is achieved through an interaction between an Arg-Gly-Asp (RGD) motif present in the penton base of the virus and cell surface integrins (mainly α_v-type). CAR expression is scarce in human kidneys.[4] Moreover, even in species in which CAR is abundantly expressed in the kidney, such as mouse or rat,[4] efficiency of renal transduction remains low. Recently, a number of strategies, aiming at enhancing the infectivity of adenovirus through re-targeting to CAR-independent pathways, have been developed.[5,6] Among them, the recombinant insertion of an RGD motif

into the fiber knob,[7] leading to binding of the virus to integrins, is particularly attractive for the setting of kidney transplantation, because integrins are widely expressed within the kidney.[8] Besides the C-terminal end of the knob, which was initially selected for insertion, the HI loop has emerged as a suitable site for genetic modification. This region is exposed on the exterior of the fiber knob, being accessible for binding to cellular targets. Moreover, the HI loop allows for insertion of relatively large sequences without affecting the structural integrity of the knob trimer.[9] Enhanced infectivity can thus be achieved, as both CAR-dependent and CAR-independent cell entry pathways can be employed.

In this study, we used a modified adenovirus that has an RGD motif inserted in the HI loop of the fiber knob as a successful strategy to enhance efficiency of adenovirus-mediated gene delivery to the transplanted kidney. The specific targeting of the interstitium, as achieved in our study, is of particular relevance for transplanted kidneys, in which the interstitium is the theatre of both rejection and fibrosis.

RESULTS

Viral titer optimization and time course of AdTL-RGD expression

Preliminary experiments showed that both AdTL-RGD and AdTL-delivered genes are optimally expressed between 48 and 72 h after infection. Initially, the optimal viral titer of AdTL-RGD was sought, taking as criteria the transduction efficiency and selectivity. The highest transduction of the transplanted kidney, measured as luciferase activity at 72 h, was found at a titer of 3×10^9 plaque-forming unit (PFU) (Figure 1). Infusion of 0.8×10^9 PFU resulted in $1.68 \times 10^4 \pm 0.58 \times 10^4$ counts per second (c.p.s)/mg protein, and 1.5×10^9 PFU yielded $1.61 \times 10^5 \pm 0.56 \times 10^5$ c.p.s/mg protein. In the 3×10^9 PFU-infused kidneys, luciferase activity was $4.91 \times 10^5 \pm 0.78 \times 10^5$ c.p.s./mg protein (30 times higher than with the lowest titer). When 6×10^9 PFU was used, no further improvement in transduction efficiency was achieved ($4.41 \times 10^5 \pm 1.03 \times 10^5$ c.p.s./mg protein). The saline-treated animals were negative for luciferase activity.

Tissue distribution of AdTL-RGD, measured as luciferase activity at 72 h in various organs, showed a similar pattern in all the infected animals. All organs studied were devoid of luciferase, except for the liver and spleen, in which very low, although dose-dependent, levels of luciferase activity were detected (Figure 1). As the 3×10^9 PFU yielded the highest and still graft-targeted transgene expression, this viral titer was considered as optimal and used to study the time course of the transgene expression. The transplanted kidney showed the highest luciferase activity at day 3, decreasing over time to day 14 after transplantation (Figure 2). All the other organs were negative for luciferase at both days 7 and 14 (data not shown).

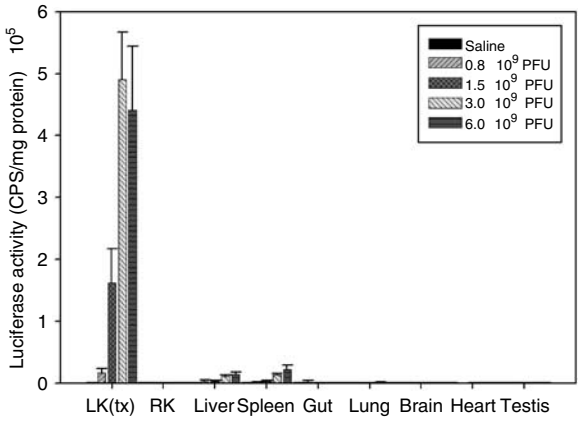

Figure 1 | Optimization of AdTL-RGD titer. Donor kidneys were infused via the renal artery, with increasing doses of AdTL-RGD ($n = 5$/group) or saline, incubated at 4°C for 20 min, flushed with saline and then transplanted into syngeneic recipients. Recipient rats were killed 3 days after transplantation and luciferase activity was measured in several organs. The best and still graft-targeted luciferase expression was achieved with 3×10^9 PFU. LK(tx) = left kidney, transplanted; RK = recipient right kidney; PFU = plaque-forming units.

Enhanced delivery of the transgene through AdTL-RGD compared to AdTL

The infection efficiency and selectivity of AdTL-RGD was compared with that of an adenovirus that lacks the RGD modification in the fiber. At 72 h after transplantation, AdTL yielded, in the transplanted kidney, less than 20% of the luciferase activity obtained with AdTL-RGD (Figure 3). Infection of the transplanted kidney with AdTL did not result in expression in all the other organs, except for the liver and the spleen, which exhibited very low levels of transgene, comparable with those yielded by AdTL-RGD (data not shown).

Highly efficient transduction of fibroblasts with AdTL-RGD

To confirm the luciferase assay data and to determine the localization of AdTL-RGD-delivered genes, expression of green fluorescent protein (GFP) was studied using immuno-

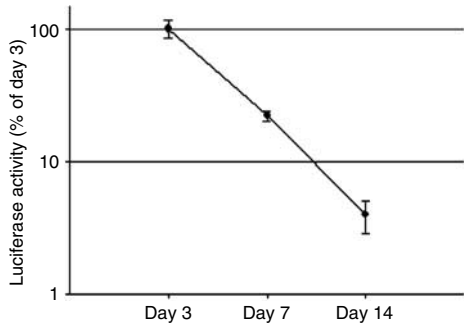

Figure 2 | Time course of AdTL-RGD-delivered gene expression in the transplanted kidneys. Donor kidneys were treated with 3×10^9 PFU AdTL-RGD before transplantation. Recipient rats were killed at 3, 7 or 14 days after transplantation infection ($n = 5$/group) and luciferase activity was measured in the transplanted kidneys. Results are presented on a logarithmic scale and expressed as percentages of luciferase activity at day 3 ± s.e.m., amounting to $4.91 \times 10^5 \pm 0.78 \times 10^5$ c.p.s./mg protein.

histochemistry. Consistent with luciferase activity data, a viral dose-dependent amount of GFP expression was found in the transplanted-infected kidneys. In all the other organs, GFP was not detectable (data not shown).

In the transplanted kidneys treated with the optimal AdTL-RGD titer (3×10^9 PFU), GFP staining was abundantly localized in interstitial cells, between the tubules, mainly in the cortex, and also in the outer medulla and to a lesser extent in the inner medulla (Figure 4a and b). Approximately 12% of the glomeruli exhibited staining for GFP (Figure 4c). No GFP expression was seen in tubular epithelial cells. Saline-treated kidneys were negative for GFP (Figure 4f). To identify the interstitial cell type, thick kidney sections were analyzed using confocal microscopy. Reconstructed three-dimensional images of the transplanted kidney clearly showed that the GFP signal was localized outside the tubular structures, in cells morphologically resembling fibroblasts (Figure 5a and b). Accordingly, double immunofluorescence for GFP and mouse anti-rat prolyl 4-hydroxylase (rPH), a marker for fibroblasts, showed colocalization of GFP- and rPH-expressing cells (Figure 5c). Furthermore, many of the transduced interstitial fibroblasts were activated fibroblasts, as shown by the double staining for GFP and mouse anti-α-smooth muscle actin (α-SMA) (Figure 5d). On the contrary, neither GFP and ectodermal dysplasia-1(ED-1), marker for macrophages, nor GFP and rat endothelial cell antigen (RECA-1), were colocalized (Figure 5e and f), ruling out the possibility that the infected cells were interstitial macrophages or interstitial endothelial cells. In the positive glomeruli, only a limited number of cells (between 3 and 17 per glomerulus) showed staining for GFP. The majority (80–90%) of the glomerular cells expressing GFP were localized in the mesangium, and proved to be mesangial cells, as shown by the colocalization of GFP and Thy-1 staining in consecutive sections (Figure 6).

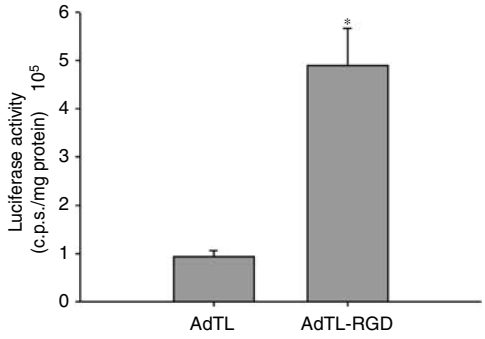

Figure 3 | Comparison between transduction efficiency with AdTL and AdTL-RGD. Donor kidneys ($n = 5$/group) were infused with 3×10^9 PFU of either AdTL or AdTL-RGD and transplanted in syngeneic rats. Luciferase activity was measured in the kidney homogenates 3 days after transplantation infection. Markedly enhanced luciferase activity was found in the transplanted kidneys when using AdTL-RGD. *$P < 0.01$.

In contrast with AdTL-RGD, and in line with luciferase data, a strongly reduced number of GFP-positive interstitial cells scattered over the kidney sections was found following infection with AdTL (Figure 4d and e). GFP-positive interstitial cells were identified as fibroblasts, using the same approach as for AdTL-RGD. However, administration of AdTL resulted in a similar amount and localization of infected cells in glomeruli (average 12.8%) as found with AdTL-RGD.

Renal (immuno)histology after transplantation infection

To determine whether AdTL-RGD and AdTL induced any additional injury in the transplanted kidneys, expression of α-SMA, an early marker of renal damage, and the presence of

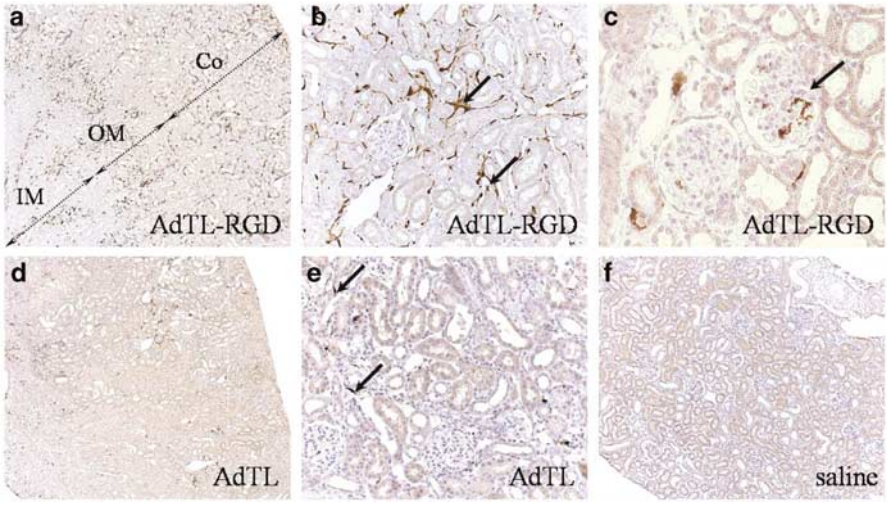

Figure 4 | Immunohistochemical staining for GFP 3 days after transplantation. Kidneys transduced with AdTL-RGD showed GFP-positive cells abundantly localized in the interstitium of the cortex (Co) and outer medulla (OM), and to a lesser extent in the inner medulla (IM) (**a**, original magnification × 40), between the tubules (**b**, arrows, original magnification × 200), and showed GFP-positive glomerulus (**c**, arrow, original magnification × 400). In contrast, only a few GFP-positive interstitial cells were found in kidneys infused with AdTL (**d**, **e**, original magnification × 40 and × 200, respectively). Saline-treated kidneys were negative for GFP (**f**, original magnification × 40). Sections were counterstained with hematoxylin.

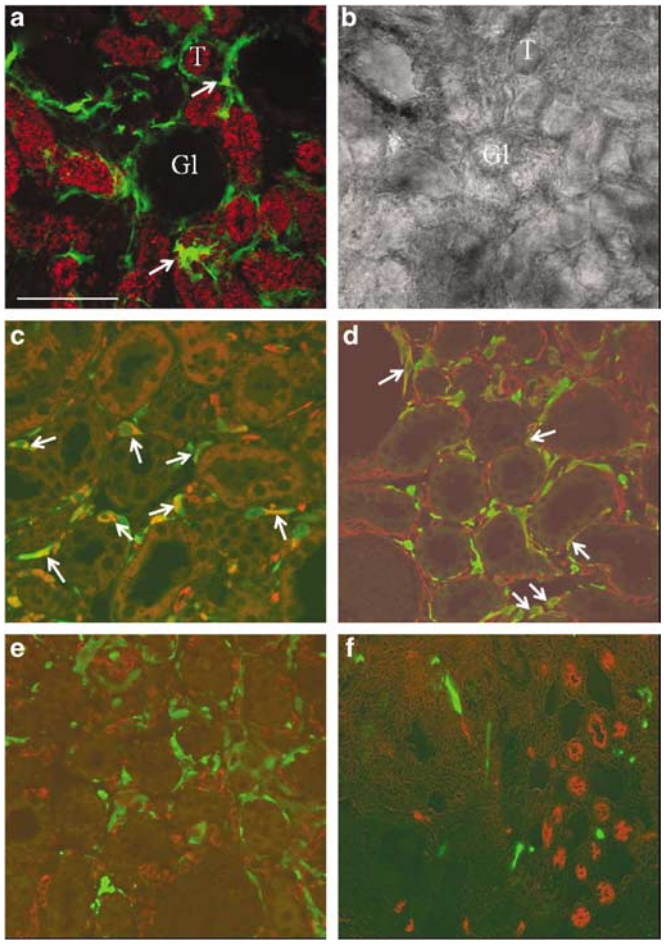

Figure 5 | Identification of interstitial cells infected with AdTL-RGD, using confocal microscopy and double immunofluorescence. (a) Three-dimensional image (30 μm thickness) of transplanted kidneys transduced with AdTL-RGD, showing fibroblast-like cells (green, arrows) abundantly localized outside the tubular structures (red, autofluorescence). (**b**) Transmission microscopy providing structural information for panel a. Representative pictures from double immunofluorescence (3 μm thickness), (**c**, colocalization, arrows) showing that the infected cells (GFP, green) were fibroblasts (rPH, red), (**d**, colocalization, arrows) many of which were activated fibroblasts expressing SMA (red). No colocalization was seen for (**e**) GFP (green) and macrophages (ED-1, red), (**f**) nor for GFP (green) and endothelial cells (RECA-1, red). Bar = 100 μm. Gl = glomerulus, T = tubule.

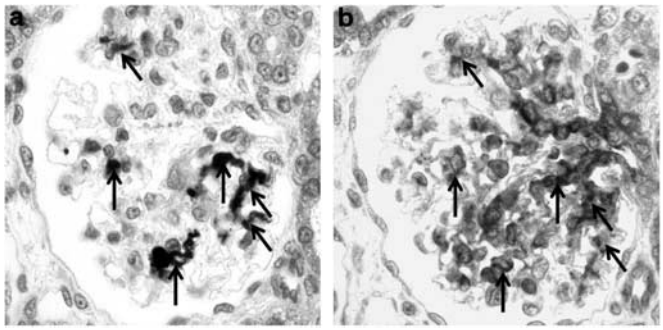

Figure 6 | Colocalization of GFP with Thy-1 in glomeruli. Consecutive sections from paraffin-embedded tissue were stained for GFP and Thy-1, a marker for mesangial cells. (**a**, black, arrows) Only a limited number of glomerular cells exhibited GFP staining. (**b**, black, arrows) Most of the cells positive for GFP had mesangial cell-like morphology and showed staining for Thy-1 in consecutive sections. Original magnification $\times$1000 for both images.

Table 1 | Quantification of immunohistological characteristics of the transplanted kidneys 3 days after transplantation infection

	Saline (*n*=4–5/ time point)	AdTL (*n*=4–5/ time point)	AdTL-RGD (*n*=5/ time point)
Interstitial α-SMA (% stained area)			
Day 3	6.54±0.85	4.91±0.83	4.09±0.73
Day 7	4.24±0.90	4.17±0.21	3.56±0.71
Day 14	2.40±0.12	2.36±0.21	2.51±0.24
Interstitial macrophages (ED-1+ cells)			
Day 3	190.8±27.7	182.6±16.6	179.7±12.6
Day 7	146.9±11.4	147.5±8.7	149.0±10.6
Day 14	90.5±15.8	98.9±10.2	82.4±13.6
Interstitial cytotoxic T lymphocytes (CD8+ cells)			
Day 3	9.5±0.9	11.9±0.5*	12.4±0.7*
Day 7	14.1±0.5	25.1±0.7*	23.4±1.3*
Day 14	11.0±0.8	17.8±1.2*	15.1±0.6*

Data are mean±s.e.m. (*$P<0.05$ compared with saline-treated kidneys). SMA= smooth muscle actin.

inflammatory cells macrophages (ED-1) and cytotoxic T lymphocytes (CD8) were evaluated 3, 7 and 14 days after the procedure and compared to saline treatment. The results are summarized in Table 1. No difference in the expression of either interstitial α-SMA or interstitial ED-1 was found between the saline- and AdTL-infused or AdTL-RGD-infused kidneys at any time point. However, an increase in interstitial CD8+ cells was detected in the virus-treated kidneys ($P<0.05$ compared to saline), irrespective of virus modification. Infiltration with CD8+ cells reached a maximum at day 7 and decreased thereafter. No immune cell infiltration (macrophages and cytotoxic lymphocytes) was present in other organs, including the liver, in animals receiving a virus-infused kidney (data not shown). This is in line with luciferase and GFP data, showing an almost complete absence of transgene expression in organs other than the transplanted kidney.

DISCUSSION

The present study demonstrates highly efficient and selective gene transfer to the transplanted kidneys using an adenovirus that has an RGD motif inserted in the HI loop of the fiber knob. Employing this strategy, enhanced infectivity of the adenovirus was achieved, without increasing virus-induced renal damage. Moreover, to our knowledge, we are the first to describe efficient transduction of the interstitial fibroblasts in a model of renal transplantation.

Previous studies have emphasized the superiority of the adenovirus compared to other vectors available for transduction of the transplanted kidney (reviewed in Van der Wouden *et al.*[2]). Depending on different parameters of graft perfusion,

such as temperature, time, pressure and viral dose, targeting to different renal compartments was achieved. However, long incubation and rather sophisticated perfusion systems were generally required in order to achieve transgene expression in the renal graft. In this study, we report a rapid and highly efficient method to transduce the transplanted kidney through an RGD-modified adenovirus. Using an intra-arterial infusion combined with clamping of the renal vein, followed by 20 min incubation at 4°C, we were able to obtain evenly distributed transgene expression in the cortex and outer medulla of the renal graft. However, the same amount of unmodified adenovirus yielded significantly less transgene expression, as shown by both luciferase activity and GFP staining. This finding is in line with previous reports, showing augmented infectivity of the RGD-adenovirus both *in vitro*[10,11] and in organs other than the kidney, in several animal models.[10–12]

Importantly, transgene expression was restricted to the renal graft. Apparently, it was mainly the specific setting of transplantation (which allowed us to flush out the viral solution before transplantation) that counted for selectivity, as the same tissue distribution pattern was observed for both adenoviral vectors. The very low levels of luciferase found in the liver and the spleen were presumably the result of a limited amount of virus recirculating from the donor kidney after transplantation.

The targeted cells within the renal graft were mainly interstitial fibroblasts and a limited number of glomerular cells. Previous studies that used recombinant adenovirus to transduce renal graft showed localization of transgene in proximal[13,14] and distal[14] tubular epithelial cells, glomeruli[15] or blood vessels.[16] The exact mechanism of targeting in each of these studies is not completely elucidated. Targeting of tubules could be a result of either glomerular filtration or the passage of adenoviral particles through the peritubular capillaries, followed by tubular infection. Glomerular transduction could be achieved by prolonged perfusion of the kidney with viral solution. Blood vessels could be infected using high pressure and prolonged incubation. Besides, species differences should also be taken into account when comparing the results of these studies. It is tempting to speculate about a mechanism of targeting in our study. Most likely, during infusion via the renal artery, the virus reaches and passes glomeruli without being filtered, as we did not see any transgene expression in the tubules. A limited amount of virus infects glomerular cells. Most of the virus reaches the interstitium, probably passing through peritubular capillaries. In the interstitium, the interaction between RGD peptide and the integrins on the surface of fibroblasts[17] is responsible for enhanced infectivity of AdTL-RGD. An alternative explanation could be that the RGD-adenovirus (and not the unmodified adenovirus) reaches the interstitium via the vasa rectae, through interaction with the integrins. However, as the vasa rectae are localized in the medulla, and as mostly the cortical area and less the medullar area was transduced, this pathway seems less plausible, or less important. Why specifically the fibroblasts were infected in our study and not other cell types that also express integrins, such as endothelial cells, is also not entirely clear. Earlier, McDonald *et al.*[18] observed selective transgene expression in the renal cortical vasculature when using an RGD insertion in the C-terminus of the fiber. Differences in infusion technique, RGD location in the virus[19] or the three-dimensional (circular or linear) structure of the RGD sequence[20] may well explain the distinct targeting in these two studies.

Interestingly, although the RGD-adenovirus infected the interstitial fibroblasts much more efficiently than the unmodified adenovirus, the percentage of infected glomeruli remained the same. As AdTL-RGD retains the CAR-binding property, it is likely that CAR expression is a limiting factor for glomerular transduction, whereas CAR-independent pathways are more important for transduction of interstitial fibroblasts. Thus, it seems conceivable that the use of a CAR-ablated, RGD-modified adenovirus would lead to specific targeting of interstitial fibroblasts. However, as the overall number of transduced glomerular cells was rather low, the use of such a truly re-targeted vector does not seem to be essentially required for a fibroblast-targeted therapeutic effect.

Targeting of fibroblasts, as achieved in our study, is of general importance for designing pathophysiologic and therapeutic studies regarding progressive renal diseases, where fibrosis is a common final pathway.[21] From previous attempts to target renal fibroblasts *in vivo*, only a few strategies were successful: ureteral injection of hemagglutinating virus of Japan liposomes,[22] injection of a large volume of naked plasmid DNA via the renal vein[23] and ureteral injection of DNA plasmids combined with electroporation.[24] However, data on fibroblast transduction in the renal graft are lacking. For kidney transplantation in particular, targeting of the interstitium is important for preventing both short- and long-term events, as the interstitium is the theater of both acute rejection and long-term fibrosis. With the first-generation adenovirus that we used in this study, transgene expression is short term, due to the immune response elicited by the virus. However, local expression of immunosupressive molecules with this vector might not only influence the rejection of the renal graft, but also inhibit the immune response against the virus itself, thereby prolonging the availability of the gene product.[25] Most suitable in this respect would probably be factors acting in a paracrine fashion, such as immunosuppressive cytokines, which would be able to modulate the tubulo-interstitial microenvironment during the first weeks after transplantation.

Proliferation and activation of the interstitial fibroblasts into α-SMA-expressing myofibroblasts is widely recognized as a key event in early transplantation-related injury, leading to fibrosis and ultimately loss of renal graft function.[26] Thus, blockade of early activation of interstitial fibroblasts might limit long-term fibrosis. In our study, many of the infected cells were SMA-expressing fibroblasts (myofibroblasts). The

SMA expression was maximal at days 3–4 and overlapped with the maximal expression of the transgene. Therefore, blockade of fibroblast activation seems feasible, through targeted expression of, for example transforming growth factor-blocking molecules (such as members of the Smad family). If extension of expression time is required, the insertion of the RGD motif in a third-generation ('gutless') adenovirus would be more suitable, as this would allow prolonged production of the transgene[27] in the interstitial fibroblasts.

Kidney transplantation is associated with marked injury due to ischemia and reperfusion. First-generation adenovirus, as used in our study, elicits both innate and adaptive immune responses and may also induce renal damage. In our study, no additional rise in either interstitial SMA expression, an early marker for renal damage, or macrophage influx was found in the virus-infused kidneys. However, an increase in cytotoxic T lymphocytes accompanied the adenoviral infection. Remarkably, enhanced transduction efficiency of AdTL-RGD did not further increase the AdTL-induced immune response, as reflected by the number of CD8 + cells. It is also noteworthy that the increased number of cytotoxic lymphocytes at later time points was not accompanied by additional renal damage, as evaluated by the amount of SMA expression, at any time point.

In summary, our current data convincingly show enhanced and selective gene transfer to the interstitial fibroblasts of a rat transplanted kidney with an RGD-modified adenovirus, allowing further design of local immunosuppressive strategies for prevention of acute rejection. For chronic renal transplant failure, an RGD-'gutless' adenovirus would probably be more suitable for approaching local immunosuppressive and/or antifibrotic therapeutic strategies.

MATERIALS AND METHODS

Vectors

First-generation recombinant adenovirus type 5 AdTL[28] and AdTL-RGD[7] were generously provided by Dr David T Curiel (University of Alabama at Birmingham, Birmingham, AL, USA). Both adenoviruses express GFP (T) and firefly luciferase under the control of a cytomegalovirus promoter, in the E1 region. The viruses were isogenic, except for the presence of RGD sequence in the AdTL-RGD. Adenoviruses were propagated on 293 cells and purified by double CsCl density centrifugation. The amount of viral particles was determined spectrophotometrically at 260 nm. The infectivity of the viruses was determined by plaque assay on 293 cells and expressed as PFU per milliliter of virus stock (PFU/ml). The viral particle/PFU ratio of the virus stocks was 100/1.

Experimental model

Inbred male Lewis rats (Lew SsnHsd, Harlan, UK) weighing 200–250 g were used. The animal experimental protocols were approved by the Animal Research Ethics Committee of the University of Groningen, The Netherlands. Experiments were performed in a rat Lewis-to-Lewis kidney transplantation model. In the donor rat, the aorta was clamped between the renal pedicles and above the bifurcation, and the left kidney was then perfused *in situ* with saline, followed by infusion of 0.4 ml of viral solution. At the time of infusion, the renal vein was clamped. At the end of infusion, the renal artery was also clamped and the kidney was stored in saline on ice for 20 min. The kidney was then slowly perfused with saline and transplanted orthotopically into a syngeneic animal, as previously described.[29] Warm ischemia time was 25 min. The right native kidney was left inside until the time the animal was killed.

In a first series of experiments, the optimal titer of AdTL-RGD for transduction of the transplanted kidney was determined. Animals were randomly divided into five groups ($n = 5$ transplanted animals/group) that received saline or 0.8×10^9, 1.5×10^9, 3×10^9 and 6×10^9 PFU, respectively. After 3 days, the rats were killed and the kidneys as well as the liver, brain, heart, lungs, spleen, gut and testis were removed and processed for luciferase assay, confocal microscopy and immunohistology. Secondly, the time course of reporter gene expression was studied, with animals being treated with the optimal AdTL-RGD titer. Two groups of animals ($n = 5$/group) were killed at days 7 and 14, and the organs were analyzed for luciferase activity. Thirdly, the suitability of AdTL-RGD-mediated gene delivery for renal transplantation was compared with that of an unmodified AdTL ($n = 4$–5/group). For this purpose, efficiency, tissue distribution and side effects were studied at days 3, 7 and 14 after transplantation. Rats that received kidneys infused with saline ($n = 4$–5/time point) were included as controls.

At the time the animals were killed, the whole body was perfused with saline and various organs were collected. One half of each organ was prepared for luciferase assay. From the other half, two slices were obtained. For confocal microscopy, one slice was fixed in paraformaldehyde–sucrose, as previously described.[30] For immunohistochemistry and immunofluorescence, the other slice was processed for paraffin embedding.

Luciferase activity assay

The luciferase activity after transduction with either AdTL-RGD or AdTL was measured using a luciferase assay kit (Luciferase Assay System, Promega Corporation, Madison, WI), according to the manufacturer's recommendations. Briefly, the tissue was homogenized in lysis buffer, incubated on ice for 1 h and centrifuged at 4°C. For luminometry, 10 μl of supernatant was added to 50 μl luciferase assay reagent and the emitted light was immediately measured using a Wallac Victor2 (Turku, Finland) luminometer. Results are expressed as counts per second/milligram protein. Protein concentration was determined by a Bradford protein assay, using bovine serum albumin as standard.

Confocal microscopy

Cells infected with GFP-expressing adenoviruses were visualized with an LSM 410 laser scanning microscope (Carl Zeiss, Jena, Germany). Confocal images of GFP fluorescence were collected using 488-nm excitation light from a two-line argon laser and a 510–525 nm band-pass filter. Simultaneously, structural information about the kidney was obtained by detection of autofluorescence at emission wavelengths higher than 570 nm. Three-dimensional information of thick tissue sections (40–60 μm) of paraformaldehyde–sucrose-fixed tissue was obtained from a stack of 30 optical sections representing 30 μm thickness.

Immunohistochemistry and immunofluorescence

To localize the transgene expression, a GFP immunostaining was performed using a rabbit anti-GFP antibody (Molecular Probes, Leiden, The Netherlands). To identify the infected cells, additional double immunostainings were performed, using markers for fibroblasts rPH (Acris Antibodies, Hiddenhausen, Germany), activated fibroblasts α-SMA (Sigma Chemical Co, St Louis, MO), macrophages (mouse anti-ED-1, Serotec Ltd, Oxford, UK), endothelial cells (mouse anti-RECA-1, generously provided by Professor G Molema, University Medical Center, Groningen, The Netherlands) and mesangial cells (mouse anti-Thy-1, a kind gift from Dr WW Bakker, University Medical Center, Groningen, The Netherlands). To evaluate the renal damage after the transplantation-infection procedure, sections were stained for SMA, macrophages (ED-1) and cytotoxic T lymphocytes (mouse anti-CD8, kindly provided by Dr JL Hillebrand, University Medical Center Groningen, The Netherlands).

Before the immunostaining procedure, paraffin sections (3 μm) were dewaxed and subjected to antigen retrieval by microwave-induced heat in 0.1 M Tris/HCl, pH 9.0 (GFP) or in 1 mM EDTA buffer, pH 8.0 (CD8), and overnight incubation in 0.1 M Tris/HCl buffer, pH 9.0, at 80°C (α-SMA, ED-1) or 0.1% protease (Thy-1).

For immunohistochemistry, a two-step immunoperoxidase technique was used, according to standard methods, as previously described.[31] Peroxidase activity was developed using 3′,3′-diaminobenzidine tetrachloride and H_2O_2. For double immunofluorescence, sections were incubated with primary antibodies (GFP and rPH, α-SMA, ED-1 or RECA-1), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies. Green (GFP) and red (rPH, SMA, ED-1 or RECA-1) fluorescent signals were visualized using confocal microscopy.

Morphometry

The number of glomeruli positive for GFP immunostaining was counted in 10 optical fields at $\times 100$ magnification. A glomerulus was considered positive if at least three cells showed GFP staining.

The expressions of α-SMA and ED-1 were measured using computer-assisted morphometry. A total of 30 (α-SMA) or 40 (ED-1) fields were evaluated at a magnification of $\times 200$. For α-SMA, the total staining was divided by the area measured, and expressed as a percentage. For ED-1, the number of positive cells per area was measured. An average score was calculated per section. CD8-positive cells were counted in 30 fields using an ocular grid at $\times 400$ magnification and expressed as an average per field.

Statistics

Results are presented as mean $\pm$ s.e.m. Statistical analyses were performed using ANOVA and Student–Newman–Keuls test. Differences were considered significant at $P < 0.05$.

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REFERENCES

1. Fischereder M, Kretzler M. New immunosuppressive strategies in renal transplant recipients. *J Nephrol* 2004; **17**: 9–18.
2. Van der Wouden EA, Sandovici M, Henning RH *et al.* Approaches and methods in gene therapy for kidney disease. *J Pharm Toxic Methods* 2004; **50**: 13–24.
3. Pellegrini C, O'Brien T, Jeppson A *et al.* Influence of temperature on adenovirus-mediated gene transfer. *Eur J Cardiac Surg* 1998; **13**: 599–603.
4. Fechner H, Haack A, Wang H *et al.* Expression of Coxsackie adenonovirus receptor and alpha$_v$-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. *Gene Therapy* 1999; **6**: 1520–1535.
5. Reynolds PN, Curiel DT. New generation adenoviral vectors improve gene transfer by coxsackie and adenoviral receptor-independent cell entry. *Kidney Int* 2002; **61**(Suppl 1): S24–S31.
6. Glasgow JN, Kremer EJ, Hemminki A *et al.* An adenovirus with a chimeric fiber derived from canine adenovirus type 2 displays novel tropism. *Virology* 2004; **324**: 103–116.
7. Dmitriev I, Krasnykh V, Miller CR *et al.* An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 1998; **72**: 9706–9713.
8. Van Goor H, Coers W, van der Horst MLC *et al.* Distribution of cytoskeletal proteins, integrins and extracellular matrix proteins in plastic-embedded human and rat kidneys. *Anal Quant Cytol Histol* 2001; **23**: 345–354.
9. Belousova N, Krendelchtchikova V, Curiel DT, Krasnykh V. Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J Virol* 2002; **76**: 8621–8631.
10. Bilbao G, Contreras JL, Dmitriev I *et al.* Genetically modified adenovirus vector containing an RGD peptide in the HI loop of the fiber knob improves gene transfer to nonhuman primate isolated pancreatic islets. *Am J Transplant* 2002; **2**: 237–243.
11. Bakker AC, van de Loo FAJ, Joosten LAB *et al.* A tropism-modified adenoviral vector increased the effectiveness of gene therapy for arthritis. *Gene Therapy* 2001; **8**: 1785–1793.
12. Reynolds PN, Dmitriev I, Curiel DT. Insertion of an RGD motif into the HI loop of adenovirus fiber protein alters the distribution of transgene expression of the systemically administered vector. *Gene Therapy* 1996; **6**: 1336–1339.
13. Zeigler ST, Kerby JD, Curiel DT *et al.* Molecular conjugate-mediated gene transfer into isolated human kidneys. *Transplant* 1996; **61**: 812–817.
14. Benigni A, Tomasoni S, Lutz J *et al.* Nonviral and viral gene transfer to the kidney in the context of transplantation. *Nephron* 2000; **85**: 307–316.
15. Heikkila P, Parpala T, Lukkarien O *et al.* Adenovirus-mediated gene transfer into kidney glomeruli using an *ex vivo* kidney perfusion system – first step towards gene therapy of Alport syndrome. *Gene Therapy* 1996; **3**: 21–27.
16. Brasile L, Stubenitsky BM, Booster MH *et al.* Transfection and transgene expression in a human kidney during *ex vivo* warm perfusion. *Transplant Proc* 2002; **34**: 2624.
17. Lygoe KA, Norman JT, Marshall JF, Lewis MP. Alpha v integrins play an important role in myofibroblast differentiation. *Wound Repair Regen* 2004; **12**: 461–470.
18. McDonald GA, Zhu G, Li Y *et al.* Efficient adenoviral gene transfer to kidney cortical vasculature utilizing a fiber modified vector. *J Gene Med* 1999; **1**: 103–110.
19. Hay CM, De Leon H, Jafari JD *et al.* Enhanced gene transfer to rabbit jugular veins by an adenovirus containing a cyclic RGD motif in the HI loop of the fiber knob. *J Vasc Res* 2001; **38**: 315–323.
20. Ogawara K, Rots MG, Kok RJ *et al.* A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells *in vitro* and *in vivo*. *Hum Gene Ther* 2004; **15**: 433–443.
21. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 1998; **339**: 1448–1456.
22. Tsujie M, Isaka Y, Ando Y *et al.* Gene transfer targeting interstitial fibroblasts by the artificial envelope-type hemagglutinating virus of Japan liposome method. *Kidney Int* 2000; **57**: 1973–1980.
23. Maruyama H, Higuchi N, Nishikawa Y *et al.* Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. *Hum Gene Ther* 2002; **13**: 455–468.
24. Nakamura H, Isaka Y, Tsujie M *et al.* Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial alpha-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rat. *Gene Therapy* 2002; **9**: 495–502.
25. Cassivi SD, Liu M, Boehler A *et al.* Transplant immunosuppression increases and prolongs transgene expression following

adenoviral-mediated transfection of rat lungs. *Exp Transplant* 2000; **19**: 984–994.
26. Badid C, Desmouliere A, Babici D *et al.* Interstitial expression of α-SMA: an early marker of chronic renal allograft dysfunction. *Nephrol Dial Transplant* 2002; **17**: 1993–1998.
27. Schiedner G, Morral N, Parks RJ *et al.* Genomic DNA transfer with high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nat Genet* 1998; **18**: 180–183.
28. Alemany R, Curiel DT. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Therapy* 2001; **8**: 1347–1353.
29. Smit-van Oosten A, Navis GJ, Stegeman CA *et al.* Chronic blockade of angiotensin II action prevents glomerulosclerosis but induces graft vasculopathy in experimental kidney transplantation. *J Pathol* 2001; **194**: 122–129.
30. Leithauser F, Trobonjaca Z, Reimann J, Moller P. *In situ* characterization of genetically targeted (green fluorescent) single cells and their microenvironment in an adoptive host. *Am J Pathol* 2001; **158**: 1975–1983.
31. Sandovici M, Henning RH, Hut RA *et al.* Differential regulation of glomerular and interstitial endothelial nitric oxide synthase expression in the kidney of hibernating ground squirrel. *Nitric Oxide* 2004; **11**: 194–200.